Beyond Oncogenesis: The Role of S-phase Kinase-associated Protein-2 (Skp2) in Vascular Restenosis

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SUMMARY

The clinical benefits of percutaneous coronary intervention, the most prevalent procedure nowadays for the treatment of symptomatic coronary artery disease, are frequently offset by the occurrence of vascular restenosis. Although the introduction of drug-eluting stents has significantly reduced restenotic rates, the rare, but potentially fatal, delayed thrombosis remains a clinical threat. Further refinement of the drug-eluting stent based on a better understanding of cell cycle regulation between the vascular smooth muscle cell (VSMC) and endothelial cell (EC) is required. In this review, we discuss the role of S-phase kinase-associated protein-2 (Skp2), previously known as an oncoprotein, in the regulation of VSMC proliferation and its signaling axis. The currently available evidence suggests that the Rac1-Skp2-p27^{Kip1} signaling axis acts as a common final pathway for many factors that regulate VSMC proliferation, such as growth factors, extracellular matrices and cyclic nucleotides. Importantly, although EC proliferation is also shown to be regulated by the same axis, cAMP seems to regulate this axis differentially between VSMC and EC, rendering the underlying mechanism of this differential regulation a promising target for the development of a new generation of drug-eluting stent. [International Journal of Gerontology 2008; 2(4): 158–166]

Key Words: coronary restenosis, cyclin-dependent kinase inhibitor p27, myocytes, rac1 GTP-binding protein, smooth muscle, S-phase kinase-associated proteins

Introduction

More than one million percutaneous coronary interventions are carried out each year worldwide[1] in patients with acute coronary syndrome or medication-refractory symptomatic coronary artery disease. The net benefits of percutaneous coronary interventions are, however, significantly diminished by the occurrence of restenosis, the rates of which are estimated to be up to 40% 6 months post-balloon angioplasty or 20% post-stenting[2,3]. Several agents, such as heparin, angiotensin-converting enzyme inhibitors, calcium–calmodulin antagonists and growth factor antagonists, have been used and demonstrated to reduce restenosis in animal studies[4,5]. Human trials, however, have generally failed to repeat their efficacy[6], reflecting the fact that targeting only a single mitogenic factor or pathway is not sufficient for treating a multifactorial disease, such as restenosis. Given that neointimal thickening is the mainstay of pathologic findings in restenotic lesions[2,3,7,8] and that cell cycle activation is the final and the essential step to enable neointimal cells to proliferate, targeting cell cycle regulators could be a promising therapeutic strategy in the prevention of restenosis[5,9]. The recent encouraging reports[10,11] showing that drug-eluting stents coated with rapamycin

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(sirolimus) or paclitaxel, which act on cell cycle regulation and were originally used in cancer treatment[12–14], were able to successfully reduce the 6-month restenosis rates to 0%, further supporting the notion that the cell cycle is an ideal and feasible target for the treatment of restenosis. However, the drug-eluting stent also has its Achilles heel, i.e., late stent thrombosis[15,16], which is thought to result from delayed re-endothelialization[17,18]. A better understanding of cell cycle regulation in different vascular cells, such as vascular smooth muscle cells (VSMC) and endothelial cells (EC), would further refine the target and allow investigation of differential cell cycle regulation in the distinct vascular cells.

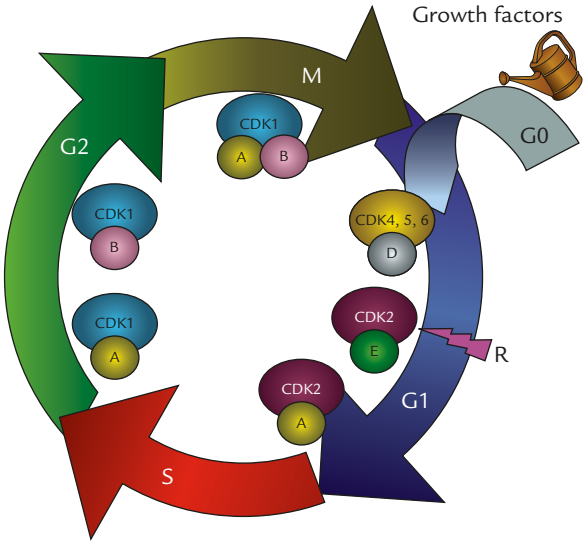

Figure 1. Overview of the cell cycle. Resting vascular smooth muscle cell remain in a quiescent state (G0). With adequate mitogenic stimulation, activated cells enter the first gap period (G1), during which the factors necessary for DNA replication for the subsequent synthetic phase (S) are assembled. After DNA replication is completed, the cells enter the second gap period (G2) to prepare for subsequent mitosis (M). The restriction point (R) is at the G1/S interphase. Before this point, the cell cycle is growth factor-dependent, and withdrawal of growth factor forces cells back to the quiescent state. Beyond this point, however, the cells acquire growth-factor independence and become committed to enter the S phase. The cell cycle is driven by a group of cyclin-dependent kinases (CDKs), the activities of which rely on the association of their distinct regulatory subunits, i.e., cyclins, such as cyclin A (A), cyclin B (B) and so forth, to form CDK–cyclin complexes.

Cell Cycle Progression

The normal cell cycle (Figure 1) is regulated by a family of serine/threonine protein kinases, called cyclin-dependent kinases (CDKs)[19], which require association with distinct regulatory subunits, namely cyclins[20], to form active holoenzymes for progression through specific points of the cell cycle. Among all CDK–cyclin complexes, the activities of CDK2, which forms the CDK2–cyclin E complex, are especially important for cells to progress through the G1 restriction point[21] (Figure 1). To tune the cell cycle progression in a more sophisticated way, the activities of cyclin–CDK complexes are further regulated by CDK phosphorylation[22] and the levels of CDK inhibitors (CDKIs)[22–25].

$p27^{Kip1}$ is probably the most important and well-studied CDKI, and was first recognized as a CDKI because of its ability to inhibit the activity of cyclin E–CDK2 and cyclin A–CDK2 complexes in cells arrested in G1 by transforming growth factor-β[26,27]. $p27^{Kip1}$ is expressed at high levels when the cells are in the quiescent (G0) state, which can be induced either by serum starvation[28] or by loss of cell adhesion[29], and is rapidly downregulated when cells enter G1. Because the activities of CDK2[27] and CDK1[30] are both inhibited by $p27^{Kip1}$, the downregulation of $p27^{Kip1}$ is crucial for both the G1/S transition (or progression through the G1 restriction point; Figure 1)[31] and G2/M progression[30,32]. Although the protein levels of $p27^{Kip1}$ oscillate during the cell cycle, $p27^{Kip1}$ messenger RNA (mRNA) levels basically do not change during an unperturbed cell cycle[33,34]; in other words, its protein levels are mainly regulated through its degradation[35].

What is Skp2?

The levels of many cell cycle effectors, such as $p27^{Kip1}$, are regulated by their degradation[36–38], which is mediated by a rapidly responsive and highly efficient mechanism called ubiquitin-mediated proteolysis[39]. Through the action of ubiquitin ligase, the protein to be degraded is first labeled with a chain of ubiquitins, a process called polyubiquitination. The labeled protein (i.e., polyubiquitinated protein) is then recognized by the "waste disposer" 26S proteasome for the subsequent breakdown[40,41] (Figure 2).

Skp1–Cul1–F-box (SCF) ubiquitin ligase (Figure 2) is one of the major ubiquitin ligases that regulate cell cycle progression, and its highly variable F-box subunit determines the substrate specificity[40,43]. S-phase kinase-associated protein-2 (Skp2) is an F-box subunit of SCF ubiquitin ligase (Figure 2), responsible for the polyubiquitination and subsequent degradation of many cell

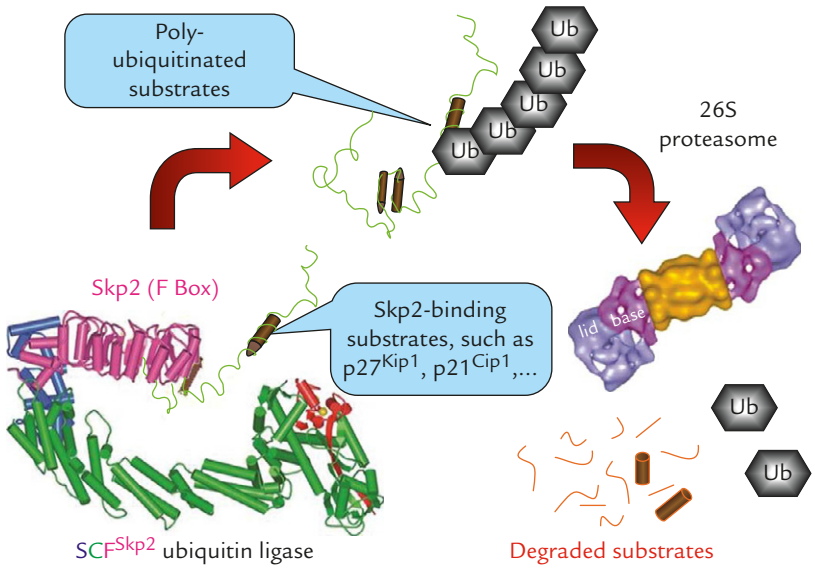

Figure 2. Skp2-mediated polyubiquitination and proteolysis. Skp2 is an F-box subunit of SCF^{Skp2} ubiquitin ligase. The SCF ubiquitin ligase is composed of three invariable subunits (scaffold), i.e., Skp1, Cul1 and Rbx, and a highly variable subunit, F-box protein, which is responsible for substrate recognition and thus determines the specificity of different SCF ubiquitin ligases. When Skp2 is in position, the Skp2-binding substrates, such as $p27^{Kip1}$, $p21^{Cip1}$ or some other cell cycle regulators, are "labeled" with multiple ubiquitins (polyubiquitinated), and the labeled proteins are subsequently degraded by the 26S proteasome. The released ubiquitins are then recycled for the next proteolytic process. The structure of the SCF^{Skp2} ubiquitin ligase is reproduced here with permission from Zheng et al.[42].

cycle regulators, including many CDKIs, e.g., $p27^{Kip1}$ [44,45], $p21^{Cip1}$ [46,47] and $p57^{Kip2}$ [48], free cyclin E[49], forkhead transcription factor FOXO1[50], mitogen-activated protein kinase (MAPK) phosphatase-1[51], etc. Although Skp2 is able to target multiple cell cycle proteins, $p27^{Kip1}$ is thought to be the most important substrate of Skp2, based on the observation that $p27^{Kip1}$ accumulates in Skp2-deficient cells[31,49], and that the phenotypic and histologic abnormalities of Skp2-deficient mice, such as small body and organ sizes, nuclear enlargement, polyploidy as well as centrosome overduplication, can be almost completely rescued in Skp2/$p27^{Kip1}$ doubly-deficient mice[30,31].

Skp2 and Oncogenesis

Knowing that Skp2 is responsible for degradation of several CDKIs, especially $p27^{Kip1}$, we are not surprised that Skp2 has been deemed an oncogene[52–54], and its overexpression, along with the related $p27^{Kip1}$ deregulation, has become the indicators of cancer progression and poor disease prognosis[55–64]. Consistent with the clinical observations, cancer cells have been found to constitutively express Skp2 and proliferate when placed in suspension[54], while non-cancer cells expressed high levels of $p27^{Kip1}$ and remained in a quiescent state[65]. Furthermore, Shim et al. also have demonstrated that transgenic mice with Skp2 exclusively expressed in the prostate gland could exhibit a low-grade prostate carcinoma[66]. Taken together, all lines of evidence point to the fact that Skp2 plays a crucial role in the regulation of cell proliferation and, thus, in cancer progression.

Skp2 and Vascular Restenosis

VSMC proliferation contributes significantly to the development of post-angioplasty or in-stent restenosis[3,8,67,68]. Although the role of Skp2 in cancer cell proliferation has been well recognized, its role in the regulation of VSMC proliferation is less well understood. We were the first to elucidate how Skp2 regulates VSMC proliferation *in vitro* and *in vivo*[69–71].

VSMCs in healthy vessels are maintained in a quiescent state[72], even in the presence of growth factors[73]. Being isolated from a vessel or when the vessels are injured, VSMCs regain the ability to proliferate and become responsive to growth factor stimulation[73,74]. We have previously demonstrated that a high $p27^{Kip1}$ level is the major reason why VSMC in the uninjured vessel

can be kept quiescent even in the presence of growth factors[73], and subsequently found that an extremely low level of Skp2 expression accounts for the abundant $p27^{Kip1}$ in the intact vessel[69]. In contrast, Skp2 expression is significantly upregulated and, thus, $p27^{Kip1}$ downregulated when the cultured VSMCs start to proliferate after serum stimulation[69,71] or when the carotid VSMCs and neointimal cells are proliferating after balloon injury[71,75]. Importantly, the kinetics of Skp2 expression perfectly mirrors that of medial and neointimal cell proliferation after balloon injury, indicating that Skp2 expression is closely related to VSMC proliferation, both *in vitro* and *in vivo*. Moreover, reports showing that adenovirus-mediated Skp2 expression is able to enhance VSMC S-phase entry even in the uninjured rat aorta[69], and that silencing Skp2 expression by Skp2 short interfering RNA in cultured VSMC reduces the S-phase entry[71], further supporting the notion that Skp2 expression is essential for VSMC proliferation. More importantly, we demonstrated that adenovirus-mediated Skp2 expression significantly enhanced neointimal thickening after filament-induced endothelial denudation in rat carotid artery[76] and that neointimal formation was significantly diminished after carotid ligation in Skp2-deficient mice as compared with the wild-type mice[77]. Taken together, the currently available evidence strongly suggests that Skp2 regulates VSMC proliferation both *in vitro* and *in vivo* and thus could play a crucial role in vascular restenosis.

Skp2 as a Common Final Pathway for VSMC Proliferation

Although a myriad of factors regulate VSMC proliferation, cell cycle regulation is the final common pathway for those factors to exert their influence on cell proliferation[5]. It would be very interesting to know if Skp2 is one of the common pathways through which the extracellular factors regulate VSMC proliferation.

Proliferation of mammalian cells, including VSMCs, is strictly regulated by the surrounding environmental factors. Both growth factor and extracellular matrix (ECM) are indispensable for cell cycle progression, and loss of either growth factor stimulation or ECM attachment generally results in G1 arrest[28,78], or even apoptosis in some susceptible cells[79]. Indeed, we have clearly shown that growth factors significantly enhance Skp2 mRNA and protein expression in VSMCs[71], and that inhibition of growth factor-related signaling, such as extracellular signal-related protein kinase, p38 MAPK and c-Jun *N*-terminal kinase, results in a reduction in VSMC Skp2 expression[80]. Furthermore, it has been demonstrated that VSMCs lose their Skp2 expression when deprived of ECM contact, and the distinct matrix attachments have resulted in different amounts of Skp2 expression, which perfectly mirrors the different effects of matrices on VSMC proliferation[69]. Focal adhesion kinase (FAK) signaling has been shown to be, at least partially, involved in this process[69].

Although positive regulators, such as growth factors and ECMs, are shown to positively regulate VSMC proliferation through the upregulation of Skp2, it would be of interest to know whether negative regulators, such as an intact endothelium, negatively regulate Skp2 expression in VSMC. An intact endothelium is able to inhibit VSMC proliferation[81,82], at least in part, through an increase in prostacyclin[83,84] and nitric oxide[84,85], which in turn increase intracellular levels of cyclic nucleotides (cyclic adenosine monophosphate [cAMP] and cyclic guanosine monophosphate [cGMP], respectively)[83,84,86]. Indeed, cAMP analogues and cAMP-elevating agents strongly inhibit Skp2 expression in VSMC both *in vitro* and *in vivo*, through a decrease in mRNA expression and an increase in protein breakdown[71]. Importantly, adenovirus-mediated ectopic Skp2 expression is found to significantly rescue cell proliferation in forskolin-(an adenylate cyclase activator) treated VSMC[71]. cGMP analogues also significantly inhibit VSMC proliferation, though not as strongly as cAMP analogues[71]. Collectively, both positive growth signals, such as growth factors or ECMs, and negative growth signals, such as cyclic nucleotides, affect VSMC proliferation through the regulation of Skp2 expression.

Do these factors have a common downstream signal to Skp2? We incidentally found that the extent of cyclic nucleotide-induced cell shape change (depicted as "stellate" or "neuron-like" cells in the literature[87]) was proportional to the inhibitory effects of cyclic nucleotides on Skp2 expression, implying that the signaling pathway regulating cell shape change could be identical to that regulating Skp2 expression. Indeed, Rac1, a member of the Rho GTPase subfamily which belongs to the small G protein superfamily and is known to be crucial for the maintenance of cell shape[88], has been demonstrated to be an important mediator upstream of Skp2[89]. This fact is supported by the evidence showing

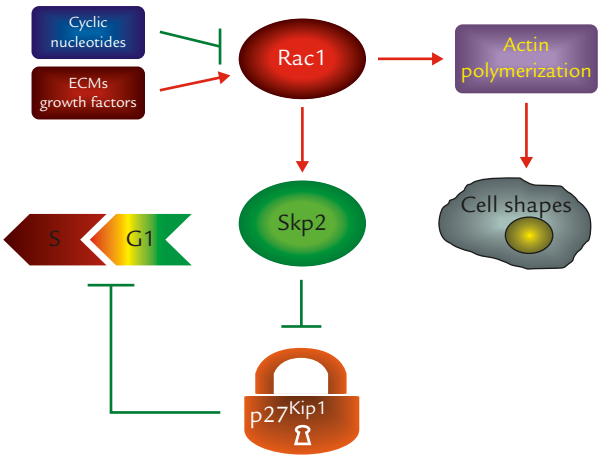

Figure 3. The Rac1-Skp2-p27^{Kip1} signaling axis. Both positive regulators (growth factors or extracellular matrices [ECMs]) and negative regulators (cyclic nucleotides) of vascular smooth muscle cell proliferation are able to upregulate and downregulate, respectively, the activity of Rac1, which is crucial for actin polymerization and thus maintaining a proper cell shape. On the other hand, Rac1 also upregulates Skp2 expression, which in turn increases the degradation of p27^{Kip1}, releases CDK2 from the inhibition, and thereby allows cells to progress through the G1/S transition. Actually, Rac1 has recently been proven to regulate Skp2 expression, at least in part, through actin polymerization[89].

that the inhibition of Rac1 signaling by the expression of dominant-negative Rac1 mutant induces the same cell shape changes and reduces Skp2 expression in VSMC. On the other hand, the expression of constitutively active Rac1 mutant is able to prevent diminution of Skp2 and reverse the cell shape changes induced by cyclic nucleotides[89]. Furthermore, vascular injury has also been demonstrated to enhance Rac1 signaling as well as Skp2 expression, and, importantly, blocking Rac1 signaling by the expression of dominant-negative Rac1 mutant is able to reduce Skp2 upregulation and the final neointimal thickening[89]. Growth factors, ECM attachment and cyclic nucleotides are all able to affect the pathways regulating Rac1 activity, and this integrated Rac1 signaling can determine the final levels of Skp2 expression and thus control VSMC proliferation (Figure 3)[80].

Skp2 in Other Cells Contributing to Vascular Pathogenesis

Apart from VSMC, Skp2 has also been found to regulate the proliferation of other cells, which may contribute to vascular pathogenesis. For example, obesity is a risk factor for the occurrence of cardiovascular events; Skp2 has been shown to control adipocyte proliferation during the development of obesity, and Skp2-deficient mice are less prone to diet-induced obesity and insulin resistance[90]. EC proliferation, in contrast to VSMC proliferation, contributes to re-endothelialization after vascular injury and thus benefits vascular repair. Bryant et al.[91] clearly showed that growth factors and ECM adhesion-related EC proliferation is mediated by the FAK-Skp2-p27^{Kip1} axis, which is similar to the control of VSMC proliferation[69].

Interestingly, although cAMP strongly inhibits Skp2 expression and VSMC proliferation, it has not been shown to significantly inhibit EC proliferation[83] and Skp2 expression in human aortic EC (Wu YJ, unpublished data, 2008). Instead, there is even evidence showing that cAMP signaling promotes EC proliferation[92–94]. The detailed mechanism of how cAMP signaling induces differential growth and Skp2 regulation between EC and VSMC is now under investigation, and further elucidation of this mechanism may provide invaluable information for the future development of a better treatment regime for vascular restenosis.

Conclusion

The Rac1-Skp2-p27^{Kip1} signaling axis plays an important role in the regulation of VSMC proliferation. Although many factors affect VSMC proliferation, the currently available evidence suggests that this axis may act as a common final pathway in VSMC to regulate cell cycle progression. Therefore, the Rac1-Skp2-p27^{Kip1} axis could be a promising target for the treatment of vascular restenosis. Furthermore, the fact that cAMP signaling regulates VSMC and EC proliferation distinctly implies that there could be a differential regulation of the Rac1-Skp2-p27^{Kip1} axis in different vascular cells. Further elucidation of this differential regulation may provide a new target for the development of more ideal drug-eluting stents, which are able to strongly inhibit neointimal thickening but without delayed or enhanced endothelial regrowth.

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